

Structural similarities of human and mammalian lipocalins, and their function in innate immunity and allergy

E. Jensen-Jarolim , L.F. Pacios , R. Bianchini , G. Hofstetter & F. Roth-Walter

Abstract

Owners and their domestic animals via skin shedding and secretions, mutually exchange microbiomes, potential pathogens and innate immune molecules. Among the latter especially lipocalins are multifaceted: they may have an immunomodulatory function and, furthermore, they represent one of the most important animal allergen families. The amino acid identities, as well as their structures by superposition modeling were compared among human lipocalins, hLCN1 and hLCN2, and most important animal lipocalin allergens, such as Can f 1, Can f 2 and Can f 4 from dog, Fel d 4 from cats, Bos d 5 from cow's milk, Equ c 1 from horses, and Mus m 1 from mice, all of them representing major allergens. The β -barrel fold with a central molecular pocket is similar among human and animal lipocalins. Thereby, lipocalins are able to transport a variety of biological ligands in their highly conserved calyx-like cavity, among them siderophores with the strongest known capability to complex iron (Fe^{3+}). Levels of human lipocalins are elevated in nonallergic inflammation and cancer, associated with innate immunoregulatory functions that critically depend on ligand load. Accordingly, deficient loading of lipocalin allergens establishes their capacity to induce Th2 hypersensitivity. Our similarity analysis of human and mammalian lipocalins highlights their function in innate immunity and allergy.

The current view on immune tolerance vs Th2 skewing of allergens

IgE-mediated allergy is characterized by a Th2-dominant immune response. The formed IgE may be directed against the genuine allergen or secondarily bind to molecules with similar epitopes on their surface, causing the phenomenon of cross-reactivity. A limited number of allergen families can initiate Th2 responses strong enough to result in IgE formation in spite of sometimes having limited amino acid homologies. In these settings, functional similarity may be a dominant feature for their Th2 induction capacity.

Consequently, it is essential to understand the very first events that actually skew the immune response to Th2. Immune cells like NKT, eosinophils (that especially in the small intestine via CD103 DC activation control Th2 priming

(1)), basophils (2), nuocytes, and NKT cells (3), but also macrophages (4) are known sources of early IL-4 and IL-13. These cytokines are released quickly upon stimulating the cells under involvement of a Notch-regulated CNS-2 enhancer (5) and the events are connected to memory in CD4 T cells (2). Disturbances of the skin and mucosal barriers and subsequent TSLP release may be crucial upstream events (6). One explanation could be that some allergens have enzymatic functions and thereby produce an epithelial danger signal (7), but many allergens have no proteolytic activity and do still exert 'adjuvant-like activation' (8). It is thus not entirely clear which danger signals go along with allergens that initiate priming or triggering of these cells to release Th2 cytokines, and would thereby mark allergens as *special*. It is noteworthy that among the known protein families in the 2009 Pfam database only 1.4% are considered allergens (9). In the case

of respiratory mammalian allergens a few protein families are absolutely dominant: lipocalins, secretoglobins and kallikreins (10), and IgE levels against them correlate with the severity of asthma (11).

Animal keeping: who is at risk?

The question of whether interactions with animals provide protection or increase risk is hotly debated, with particular points of discussion being the subject's age at time of exposure, and the exact animal species or breed. In German-speaking Europe between 16% and 25% of households own cats, and 12% dogs (Table 1), with an estimated dark number of plus 30%. The interrelation between people and domestic animals has thus considerably changed in the last decades. Humans and pets in Western societies are cohabitating more close and in a cleaner way than ever before, a development which is associated with a good economy. Simultaneously, the rates of allergies and asthma in human patients caused by animal dander are increasing (12). In a cohort study of 696 school children 259 were sensitized to animals, and the majority of them to multiple species (13). The higher awareness today of allergy risk associated with pet ownership and the desire for nonfurry pet animals go hand in hand, introducing novel, alternative allergen threats into homes such as those presented from feed insects for reptiles (14). However, pets may also shuttle traditional allergens into homes: house dust mites can be identified in bedding, skin and hair coats of dogs (15). Interestingly, also pet animals and horses increasingly suffer from atopic eczema and other forms of allergies (16, 17). Therefore, it might be important to look at the problem bi-directionally and watch for systematic problems in our society and environment.

In contrast to the earlier trend to minimize allergen release by washing pets (18), most recent work suggests that the introduction of pets into homes early in life is protective (19). It has further been shown that lowering pet exposure may reduce the IgE and symptom levels if allergy is established (20). The interplay of allergens and the microbiome may be decisive for inducing hypersensitivity: Low levels of Firmicutes and Bacteroidetes in the skin flora concomitant with high allergen exposure enhance the risk for atopy and

asthma (21). Needless to say that forced hygiene, washing and disturbance of the skin pH, enzymatic shampooing (7), and even the water quality (22), may impact the physiologic skin flora (23). In some studies cat and dog ownership did not elevate the risk of asthma (24), which might be correlated with exposure to the associated 'good' microbiomes of the animals. However, people with dog allergy more seldom keep dogs (25).

The observation that there are breed-specific differences in eliciting symptoms has initiated the search for 'hypoallergenic' cats, dogs, and horses and stimulates breeders to single out mutants. The experimental evidence, however, is sparse and this topic needs to be addressed in its whole complexity regarding the countless breeds of domestic animals. The situation gets even more complicated as an animal's age and gender also play a role in allergies to them (26). Here, molecular diagnosis has significantly contributed to important practical recommendations (19), including the neutering, or avoidance of male animals (27, 28). Whereas the expectations were that hypoallergenic animals would shed less allergens, the contrary turned out to be the case: in a recent study putative hypoallergenic dogs produced higher levels of Can f 1, a lipocalin allergen (29).

Comparing animal, human, and other lipocalins

What is not yet understood is why allergens in particular provoke a Th2 shift in the immune response and lead to IgE production. Why exactly, are lipocalins so important elicitors of allergies to animals even though humans also do express lipocalins, a fact that is maybe less known to the allergist?

Lipocalins owe their name to the fact that they usually carry lipids (or other hydrophobic compounds) accommodated in a large, calyx-like cavity formed by their characteristic β -barrel fold (Fig. 1). Ligand specificity of lipocalins is regulated by the amino acid residues that line their binding pocket. A large variety of ligands, most of them hydrophobic or amphiphilic, binds to lipocalins that include lipids, steroids, hormones as well as metabolites such as vitamins, cofactors, and odorants. In some cases, binding of the ligand is critical for the biological functions of the lipocalin.

Exploring the PDB by entering the query 'lipocalin', renders nearly 300 experimental structures available for lipocalins belonging to 24 eukaryotic species and 10 bacteria species of which 79 structures correspond to humans (Table 2).

However, these numbers include mutants, engineered variants and the same protein in complex with different ligands, particularly for the most studied cases such as bovine, murine, and human lipocalins.

Allergenic lipocalins have been especially identified in furry animals (19), for instance in dogs: Can f 1, -2, -4, -6, cats: Fel d 4, -7, mice: Mus m 1, horses: Equ c 1, guinea pigs: Cav p 2, -3 (30), and hamsters (31). Lipocalins are also found in tick saliva (32) where they sequester histamine and thereby interfere with its signaling via H1 receptor (32), and contribute to camouflaging the bite. Importantly, humans also express 'a menagerie of lipocalins' (33), such as LCN2 (alternatively called siderocalin/SCN; neutrophil gelatinase-associated

Table 1 Statistical comparison of percentage and numbers of animals in German-speaking households (Statistics Germany <http://www.wissenswertes.at/index.php?id=haustiere-statistik>)

Species	Austria (%)	Switzerland (%)	Germany (%)	Numbers in Germany (Mio.)
Cats	25.6	25	16.5	8.2
Dogs	30	12	13.3	5.4
Rodents		11	6.4	5.6
Birds		7	4.9	3.4
Aquaria		6	4.4	2.0
Ponds			4.0	2.1
Terraria			1.2	0.4

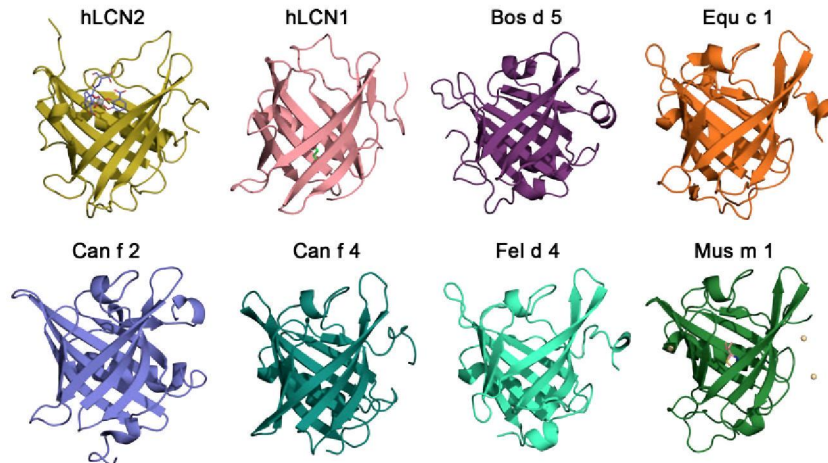


Figure 1 Gallery of human and animal lipocalins. PDB codes for experimental structures are given in parentheses. hLCN2: human LCN2 in complex with the siderophore enterobactin shown as sticks chelating the central Fe^{3+} ion shown as a gray sphere (1L6M (82)). hLCN1: human tear lipocalin in complex with 1,4-butanediol (3EYC (83)). Bos d 5: bovine β -lactoglobulin unliganded form (3NPO (84)).

Equ c 1: major horse allergen (1EW3 (85)). Can f 2: dog allergen (3L4R (45)) with IgE cross-reactivity with the cat allergen Fel d 4. Can f 4: dog dander allergen (4ODD (86)). Fel d 4: cat allergen, homology model structure based on template Equ c 1. Mus m 1: pheromone binding rodent major urinary protein in complex with 2-(sec-butyl)-thiazole and Cd^{2+} ions shown as yellow spheres (1MUP (87)).

lipocalin/NGAL, 24p3), or human LCN1 (alias tear lipocalin, TLC). A gallery of human and animal lipocalins for which experimental structures exist is represented in Fig. 1. Their well-recognized characteristic β -barrel fold has emerged in the last few years as a successful molecular scaffold to engineer novel binding proteins, so called anticalins, with human lipocalins playing a particularly relevant role in clinical applications (33). In an innovative approach four different canine lipocalins were recently linked into one multimeric molecule which indeed induced IgG upon vaccinating mice and which therefore may be used for vaccination against dog allergy in the future (34).

Lipocalins occur in secretions such as saliva, in urine (besides kallikreins) (28), and shed skin, which are all important sources for the release of allergens into our environment (35). Taken the intense interactions between owner and animal it is plausible that there is a bidirectional exchange of allergens, as it is the case with phylotypes of the microbiome of human dog owners and dogs (36).

Given their importance, many lipocalins of domestic animals and pets have been cloned and their production improved (such as Can f 1, -2, or -4 (37–40)), but methods were also developed for the quantification of the natural allergens to be able to evaluate the exposure levels in different environments, e.g., for Can f 4 (41) which is important for over 80% of people with dog allergies for IgE binding. Such measurements have recently brought up the fact that so-called hypoallergenic dogs shed even higher levels of Can f 1 into homes (29).

Comparing structural features of lipocalins

Lipocalins display a well-known overall similarity in their fold in spite of the relatively low degree of amino acid sequence

identities of below 25% (42). This fold is characterized by a calyx-shaped cavity within the central β -barrel formed by eight antiparallel β -strands βA – βH (in what follows, labels refer to Fig. S1). Loops around the cavity regulate ligand access to the calyx binding site. The extended loop L1 which comprises a $3_{10}(2)$ helix together with hairpin loops L3, L5, and L7 define the top wider end of the barrel, whereas hairpin loops L2, L4, and L6 make the bottom narrower end. This arrangement settles a topology of the β -barrel which is one of the distinctive features of lipocalins. This fold also presents a conserved N-terminal $3_{10}(1)$ helix as well as a medium-size (about 10 residues) conserved α helix H1 and an additional β -strand, βI , near the C-terminal segment. Some lipocalins (e.g., bovine lactoglobulin) display additional secondary structure elements such as a small N-terminal β -strand (β) and a C-terminal $3_{10}(3)$ helix. The presence of one to three structurally conserved regions (SCR1–SCR3) associated to particular local arrangements of strands and helices have been used to divide lipocalins into two structural subfamilies termed kernel lipocalins (those having the three SCRs) and outlier lipocalins (those having a maximum of two SCRs). SCR1 comprises $3_{10}(1)$ and βA in the N-terminal region, SCR2 is composed of sections of βF and βG together with loop L6, and SCR3 is defined by the end of βH together with adjacent residues of H1 in the C-terminal region (Fig. S1). Obviously, amino acid residues in loops L1, L3, L5, and L7 lining the binding site regulate the ligand specificity of lipocalins. Although the name of this protein family makes explicit mention of lipophilic (hydrophobic) compounds, there is a large variety of ligands including amphiphilic molecules or compounds with hydrophobic segments also bearing polar groups that bind to lipocalins.

All these structural similarities observed in these proteins could help to understand IgE cross-reactivities (43) between animal and human lipocalins, and between lipocalins from

Table 2 Organisms with experimental structure of lipocalins available in the Protein Data Bank

Organism		Organism	
Eukaryota	Number of entries	Bacteria	Number of entries
<i>Anguilla japonica</i>	3	<i>Bacillus pumilus</i>	1
<i>Arabidopsis thaliana</i>	2	<i>Bacillus subtilis</i>	1
<i>Argas monolakensis</i>	4	<i>Bacteroides eggerthii</i>	1
<i>Argas reflexus</i>	2	<i>Bacteroides ovatus</i>	1
<i>Bos taurus</i>	37	<i>Bacteroides thetaiotaomicron</i>	2
<i>Canis lupus familiaris</i>	2	<i>Bacteroides uniformis</i>	3
<i>Capra hircus</i>	3	<i>Bradyrhizobium diazoefficiens</i>	3
<i>Coturnix coturnix</i>	1	<i>Enterococcus faecalis</i>	1
<i>Coturnix japonica</i>	2	<i>Escherichia coli</i>	6
<i>Diploptera punctata</i>	3	<i>Escherichia coli K-12</i>	2
<i>Equus caballus</i>	1	<i>Helicobacter pylori</i>	1
<i>Homarus gammarus</i>	4	Total	22
<i>Homo sapiens</i>	79		
<i>Lingulodinium polyedrum</i>	1		
<i>Mesocricetus auratus</i>	1		
<i>Mus musculus</i>	34		
<i>Ornithodoros moubata</i>	4		
<i>Ovis aries</i>	2		
<i>Pieris brassicae</i>	5		
<i>Rattus norvegicus</i>	4		
<i>Rhipicephalus appendic</i>	6		
<i>Rhodnius prolixus</i>	68		
<i>Sus scrofa</i>	6		
<i>Trichosurus vulpécula</i>	3		
Total	277		

As of June 22, 2015, the search for 'lipocalin' in the PDB yielded 299 entries distributed between eukaryota and bacteria.

different animals (44–46), although not necessarily correlating with the symptoms.

For instance, in the current view at the amino acid level the dog allergens Can f 1 and Can f 2 correspond to human LCN1 and -2, respectively, whereas the four molecules only share 25% amino acid similarities between them. The amino acid sequences of various animal and human lipocalins LCN1 and -2 are aligned in Fig. 2. Identities above 60% occur between Fel d 4 and Equ c 1, Fel d 4 and Can f 6, Ory c 4, Rat n 1 and Mus m 1, and Can f 1 and Fel d 7.

In contrast, there was no amino acid homology among human LCN2 and any of the analyzed animal lipocalins, only 57% among human LCN1 and Fel d 7, and 56% among LCN1 and Can f 1.

T-cell responses have been found to be 'suboptimal' for Can f 1 (47), although resembling those to human LCN1 (48). Can f 4 T-cell responses were shown to be a Th2-deviated memory response, in part to peptides with promiscuous HLA-binding capacity (49), whereas the DR4-DQ8 haplotype and T-cell receptor Vbeta T-cell subset were indicative for the absence of Can f 1 allergy (50).

The above implies that amino acid sequence identities alone do not sufficiently explain why all lipocalins are important in innate immune regulation, and why they are typically allergenic. The known anti-bacterial activity of the human neutrophil lipocalin LCN2 (NGAL) is caused by the high affinity of this lipocalin for the siderophore-Fe3+ complex

and it should be investigated systematically whether from this it can be extrapolated to animal lipocalins. In fact, the molecular ligand transport function of lipocalins and of lipocalin-like proteins could be particularly significant for the Th2 skewing capacity, independent of their mammalian (51) or plant (52) origin. We propose that this feature is likely connected with the similar β -barrel fold in human and animal lipocalins (Fig. 3) (33), as well as with the binding plasticity of their calyx-like cavity able to accommodate a great disparity of substances involved in metabolism and communication (33, 39, 53). Major sources of (at least the human) LCN2 are the liver (54) and macrophages (55), with sphingosin-1-phosphate inducing its production. Lipocalins are elevated and released during inflammation (56), infections (57) and sepsis (58–60), especially when the urinary tract is affected in humans and dogs (61, 62); in cardiovascular inflammation they get overexpressed in the brain and linked with depression (63); in cancer LCN2 is overexpressed (11, 64) prompting interaction with metalloproteinase-9 (MMP-9) (65), but only LCN2 is prognostic (66). In the immunosuppressive environment of the tumor stroma LCN2 cooperates with CCL2 to induce immunoregulatory DCs and subsequently CD4(+)FoxP3(+) Treg cells and supports metastasis (67). Moreover, local IL-10 stimulates tumor infiltrating M2 macrophages to release LCN2 and iron (68, 69). Hence, in cancer the opposite of what is needed in allergy is the problem (70). It is further known that macrophages convert to an

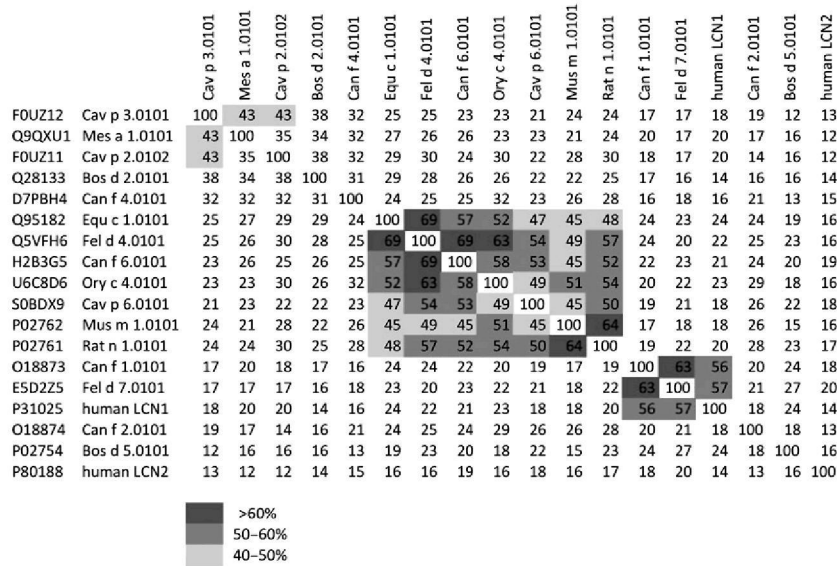


Figure 2 Comparison of human lipocalins and major animal lipocalin allergens. Amino acid identities (%) between mammalian lipocalin allergens and human lipocalin 1 and 2 were determined with Clustal Omega via <http://www.ebi.ac.uk/Tools/msa/clustalo/>. High-

lighted regions show identities higher than 40%. The different gray shades illustrate the levels of amino acid identities. Amino acid sequences were retrieved from UniProtKB database (<http://www.uniprot.org/uniprot/>).

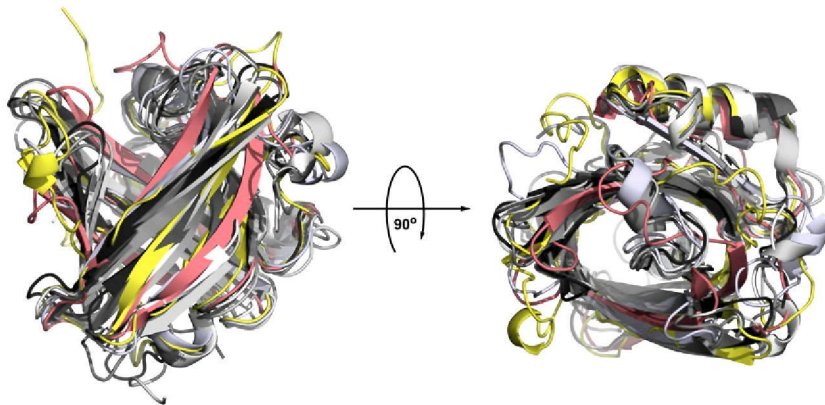


Figure 3 Symphony of human and animal lipocalins by structural superimposition. Human LCN2 and LCN1 lipocalins are colored yellow and salmon, respectively, Bos d 5 is colored blue white and

the remaining lipocalins in Fig. 1 are colored in different shades of gray from white (Equ c 1) to black (Mus m 1). The rotated image on the right gives an upper view of the calyx-like cavity.

anti-inflammatory type by IL10 and glucocorticoid stimulation (71). Alternatively activated macrophages further regulate the immune response by secreting LCN2, which is directly linked with IL10 formation (and in our own hands glucocorticoid release). Taken together, lipocalins are regarded as biomarkers for several diseases (72) and are at the crossing point between immunity and tolerance that is of particular interest for allergen immunotherapy.

The secret function of lipocalins determined by their pocket load

Some important human lipocalins are considered acute phase proteins with innate immune function, especially against

gram-negative bacteria. Their growth depends on iron, which they secure via the secretion of a bacterial siderophore, [= a high-affinity iron acquisition molecule], like enterobactin, an iron-specific chelating agent closely related to the ligand in the crystal structure of human LCN2 displayed in Fig. 1. We speculate that, like LCN2 which withdraws iron from bacteria by accepting it into their own pocket, also some of the other mammalian lipocalins may act bacteriostatically (Fig. 3) (73). On the other side of this evolutionary interplay, bacterial siderophores can inhibit the function of neutrophil myeloperoxidase and enhance the bacterial survival (74), or simply overwhelm the lipocalin defense system (75). A lipocalin-2 receptor (termed 24p3/NGAL) and Lipoprotein Receptor-Related Protein have been described binding LCN in

humans and rodents (65, 76), possibly also involving MMP9 in a triple interaction.

Less attention has been given to a potentially differential immune function of lipocalins depending on the load state of their pocket, either holo – full, or apo – empty. Interestingly, the major birch pollen allergen Bet v 1 has a relation to bacterial proteins (77) by its old scaffold and a central pocket, which was predicted to bind quercetin-3-*O*-sophorose (78). However, the flavonoid quercetin also behaves as a siderophore (52). By means of an *in silico* approach using flexible docking calculations and geometry optimizations we were able to analyze the optimal binding of human LCN2 to several siderophore-iron complexes with 1, 2, or 3 catechol chelating moieties (79). This study allowed us to estimate protein-ligand dissociation constants in the micro- to nanomolar range indicative of a high affinity that arises to a great extent from the electrostatic interactions between amino acid residues surrounding the outer part of the cup-like cavity and polar groups of the siderophore (79). A further illustration of this optimal binding is depicted in Fig. 4 where the model complex between hLCN2 and a hexadentate iron chelator, Fe(DHBA)₃, is shown. Note that the presence of hydrophobic moieties and polar groups in the ligand is consistent with the presence of hydrophobic residues together with charged (mainly basic) side chains in the protein that produce the electrostatic effects largely responsible for this efficient protein-ligand binding. However, we emphasize that more experimental studies have to be under-

taken not only to approve our estimation with certainty *in vitro*, but also to be able to extrapolate to other lipocalin allergens.

More importantly, the load of this binding pocket is decisive for the immunomodulatory function of lipocalins. Only the apo-form of lipocalin Bos d 5 and lipocalin-like allergen Bet v 1 (51, 52) promoted the survival of CD4⁺ T cells and IL-13 production in human PBMCs, whereas the holo-form was nonallergenic, so far at least *in vitro*. Consistent with this function, the loading state of brain lipocalin is decisive for the survival of neuronal cells (80). Furthermore, in a dose-dependent loading approach, holo-LCN2 had the highest capacity to induce HLA-G(+)/FoxP3(+) T-regulatory cells (81). Therefore, it may be anticipated that exogenous lipocalins, animal allergens, may be tolerated rather than inducing allergy, when complexed with ligands.

Synopsis

Humans and their domestic animals share molecules that play a role both physiologically in innate immunity, and pathologically as allergens. Whereas many important animal allergens relevant for humans belong to the lipocalin family, it is not known yet whether *vice versa* human lipocalins may act as allergens for pet animals. It is not by accident that the lipocalin fold is highly conserved in the animal kingdom. It allows lipocalins to withdraw iron from microbes into their molecular pocket and thereby act bacteriostatic.

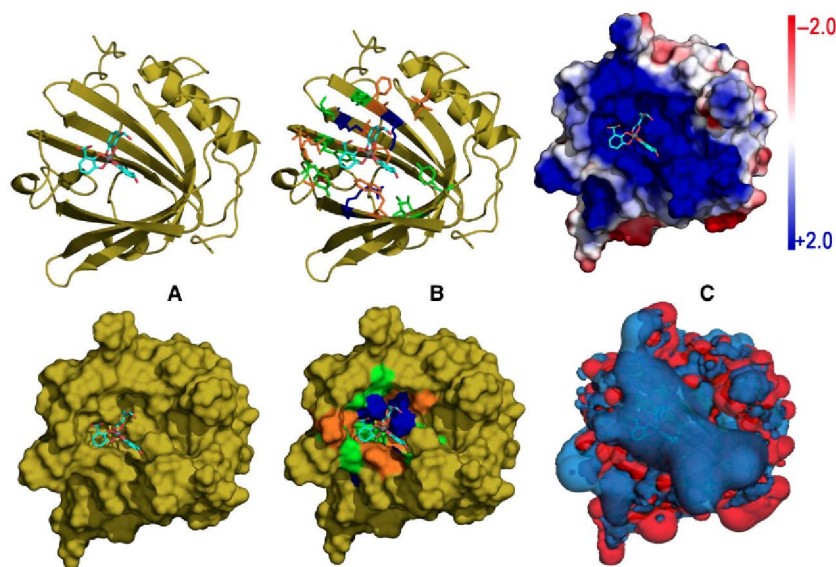


Figure 4 Prototype of lipocalin-siderophore binding: model complex of human LCN2 and Fe(DHBA)₃, a hexadentate iron-chelator. The siderophore (DHBA = 2,3-dihydroxybenzoic acid) is depicted as sticks with carbons in cyan, oxygens in red, and Fe³⁺ ion as a gray sphere. (A) Geometry of the siderophore in the cup-like cavity (top) and the corresponding protein surface showing the topography of the pocket (bottom). (B) Residues within a 5 Å distance from the ligand color-coded as follows: basic = blue, polar = green, and nonpolar = orange. Top: ribbon and sticks

diagram. Bottom: the corresponding color-coded surface. (C) Poisson-Boltzmann (PB) electrostatic potential of hLCN2. Top: PB potential mapped onto the protein surface according to the scale indicated in the bar. The strongly positive electrostatic nature of the pocket area stabilizes the electrically negative oxygenated groups in the siderophore. Bottom: +2 (blue) and -2 (red) 3D isosurfaces of the PB electrostatic field created by hLCN2. A large positive isosurface covers the cavity area in which the siderophore is accommodated.

In conclusion lipocalins may, independent of their mammalian other animal, or plant origin, house siderophore ligands, which critically determine their innate immunomodulatory as well as allergenic characteristics. It has been shown that in loaded state human lipocalins induce regulatory T cells, whereas empty lipocalins rather promote Th2 responses and inflammation. To this end, the interplay between exogenous lipocalins and human LCNs is not resolved, but it is tempting to speculate that they interfere with each other either via receptor binding or via capturing each other's iron ligand.

formed calculations, computer modeling, and structural alignments of all structures of this study (Figs 1, 3 and 4). Bianchini R and Hofstetter G involved in amino acid sequence searches and comparisons and contributed to the writing of the manuscript; Hofstetter G also contributed to the design of Fig. 2. Pacios, LF, and Roth-Walter, F contributed to the overall scientific concept and writing of the manuscript.

References

1. Chu DK, Jimenez-Saiz R, Verschoor CP, Walker TD, Goncharova S, Llop-Guevara A et al. Indigenous enteric eosinophils control DCs to initiate a primary Th2 immune response in vivo. *J Exp Med* 2014;**211**:1657–1672.
2. Khodoun MV, Orekhova T, Potter C, Morris S, Finkelman FD. Basophils initiate IL-4 production during a memory T-dependent response. *J Exp Med* 2004;**200**:857–870.
3. Camelo A, Barlow JL, Drynan LF, Neill DR, Ballantyne SJ, Wong SH et al. Blocking IL-25 signalling protects against gut inflammation in a type-2 model of colitis by suppressing nuocyte and NKT derived IL-13. *J Gastroenterol* 2012;**47**:1198–1211.
4. Byers DE, Holtzman MJ. Alternatively activated macrophages and airway disease. *Chest* 2011;**140**:768–774.
5. Tanaka S, Tsukada J, Suzuki W, Hayashi K, Tanigaki K, Tsuji M et al. The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. *Immunity* 2006;**24**:689–701.
6. Salter B, Oliveria J, Nusca G, Smith S, Watson R, Comeau M et al. Thymic stromal lymphopoietin activation of basophils in patients with allergic asthma is IL-3 dependent. *J Allergy Clin Immunol* 2015;**136**:1636–1644.
7. Stremnitzer C, Manzano-Szalai K, Willensdorfer A, Starkl P, Pieper M, Konig P et al. Papain degrades tight junction proteins of human keratinocytes in vitro and sensitizes C57BL/6 mice via the skin independent of its enzymatic activity or TLR4 activation. *J Invest Dermatol* 2015;**135**:1790–800.
8. Osterlund C, Gronlund H, Gafvelin G, Bucht A. Non-proteolytic aeroallergens from mites, cat and dog exert adjuvant-like activation of bronchial epithelial cells. *Int Arch Allergy Immunol* 2011;**155**:111–118.
9. Breiteneder H. Protein families: implications for allergen nomenclature, standardisation and specific immunotherapy. *Arb Paul Ehrlich Inst Bundesinstitut Impfstoffe Biomed Arzneim Langen Hess* 2009;**96**:249–254.
10. Aalberse RC. Mammalian airborne allergens. *Chem Immunol Allergy* 2014;**100**:243–247.
11. Wu B, Li C, Du Z, Yao Q, Wu J, Feng L et al. Network based analyses of gene expression profile of LCN2 overexpression in esophageal squamous cell carcinoma. *Sci Rep* 2014;**4**:5403.
12. Morris DO. Human allergy to environmental pet danders: a public health perspective. *Vet Dermatol* 2010;**21**:441–449.
13. Bjerg A, Winberg A, Berthold M, Mattsson L, Borres MP, Ronmark E. A population-based study of animal component sensitization, asthma and rhinitis in schoolchildren. *Pediatr Allergy Immunol* 2015;**26**:557–63.
14. Jensen-Jarolim E, Pali-Schöll I, Jensen S, Robibaro B, Kinaciyan T. Caution: reptile pets shuttle grasshopper allergy and asthma into homes. *World Allergy Organ J* 2015;**8**:24.
15. Jackson A, Foster A, Hart B, Helps C, Shaw S. Prevalence of house dust mites and dermatophagoides group I antigens collected from bedding, skin and hair coat of dogs in south-west England. *Vet Dermatol* 2005;**16**:32–38.
16. Jensen-Jarolim E, Einhorn L, Herrmann I, Thalhammer JG, Panakova L. Pollen allergies in humans and their dogs, cats and horses: differences and similarities. *Clin Transl Allergy* 2015;**5**:15.
17. Mueller R, Janda J, Jensen-Jarolim E, Rhyner C, Marti E. Allergens in veterinary medicine. *Allergy* 2015;**71**:23–31.
18. Avner DB, Perzanowski MS, Platts-Mills TA, Woodfolk JA. Evaluation of different techniques for washing cats: quantitation of allergen removed from the cat and the effect on airborne Fel d 1. *J Allergy Clin Immunol* 1997;**100**:307–312.
19. Konradsen JR, Fujisawa T, van Hage M, Hedlin G, Hilger C, Kleine-Tebbe J et al. Allergy to furry animals: new insights, diagnostic approaches, and challenges. *J Allergy Clin Immunol* 2015;**135**:616–625.
20. Erwin EA, Woodfolk JA, James HR, Sattinover SM, Platts-Mills TA. Changes in cat specific IgE and IgG antibodies with decreased cat exposure. *Ann Allergy Asthma Immunol* 2014;**112**:545–550.
21. Lynch SV, Wood RA, Boushey H, Bacharier LB, Bloomberg GR, Kattan M et al. Effects of early-life exposure to allergens and bacteria on recurrent wheeze and atopy in urban children. *J Allergy Clin Immunol* 2014;**134**:593–601.
22. Tanaka A, Matsuda A, Jung K, Jang H, Ahn G, Ishizaka S et al. Ultra-pure soft water ameliorates atopic skin disease by

- preventing metallic soap deposition in NC/Tnd mice and reduces skin dryness in humans. *Acta Derm Venereol* 2015;**95**:787–91.
23. Belkaid Y, Segre JA. Dialogue between skin microbiota and immunity. *Science* 2014;**346**:954–959.
 24. Ezell JM, Wegienka G, Havstad S, Ownby DR, Johnson CC, Zoratti EM. A cross-sectional analysis of pet-specific immunoglobulin E sensitization and allergic symptomatology and household pet keeping in a birth cohort population. *Allergy Asthma Proc* 2013;**34**:504–510.
 25. Medjo B, Atanaskovic-Markovic M, Nikolic D, Spasojevic-Dimitrijeva B, Ivanovski P, Djukic S. Association between pet-keeping and asthma in school children. *Pediatr Int* 2013;**55**:133–137.
 26. Smallwood J, Ownby D. Exposure to dog allergens and subsequent allergic sensitization: an updated review. *Curr Allergy Asthma Rep* 2012;**12**:424–428.
 27. Basagana M, Bartolome B, Pastor-Vargas C, Mattsson L, Lidholm J, Labrador-Horrillo M. Involvement of Can f 5 in a case of human seminal plasma allergy. *Int Arch Allergy Immunol* 2012;**159**:143–146.
 28. Mattsson L, Lundgren T, Everberg H, Larsson H, Lidholm J. Prostatic kallikrein: a new major dog allergen. *J Allergy Clin Immunol* 2009;**123**:362–368.
 29. Vredegoor DW, Willemse T, Chapman MD, Heederik DJ, Krop EJ. Can f 1 levels in hair and homes of different dog breeds: lack of evidence to describe any dog breed as hypoallergenic. *J Allergy Clin Immunol* 2012;**130**:904–909.
 30. Hilger C, Swiontek K, Kler S, Diederich C, Lehnert C, Vogel L et al. Evaluation of two new recombinant guinea-pig lipocalins, Cav p 2 and Cav p 3, in the diagnosis of guinea-pig allergy. *Clin Exp Allergy* 2011;**41**:899–908.
 31. Torres JA, de Las Heras M, Maroto AS, Vivanco F, Sastre J, Pastor-Vargas C. Molecular and immunological characterization of the first allergenic lipocalin in hamster: the major allergen from Siberian hamster (*Phodopus sungorus*). *J Biol Chem* 2014;**289**:23382–23388.
 32. Valdes JJ. Antihistamine response: a dynamically refined function at the host-tick interface. *Parasit Vectors* 2014;**7**:491.
 33. Schiefner A, Skerra A. The menagerie of human lipocalins: a natural protein scaffold for molecular recognition of physiological compounds. *Acc Chem Res* 2015;**48**:976–985.
 34. Nilsson OB, Neimert-Andersson T, Bronge M, Grundstrom J, Sarma R, Uchtenhagen H et al. Designing a multimer allergen for diagnosis and immunotherapy of dog allergic patients. *PLoS One* 2014;**9**:e111041.
 35. Polovic N, Waden K, Binnmyr J, Hamsten C, Gronneberg R, Palmberg C et al. Dog saliva – an important source of dog allergens. *Allergy* 2013;**68**:585–592.
 36. Song S, Lauber C, Costello E, Lozupone C, Humphrey G, Berg-Lyons D et al. Cohabiting family members share microbiota with one another and with their dogs. *Elife* 2013;**2**:e00458.
 37. Kamata Y, Miyanomae A, Nakayama E, Miyanomae T, Tajima T, Nishimura K et al. Characterization of dog allergens Can f 1 and Can f 2. 2. A comparison of Can f 1 with Can f 2 regarding their biochemical and immunological properties. *Int Arch Allergy Immunol* 2007;**142**:301–308.
 38. Konieczny A, Morgenstern JP, Bizinkauskas CB, Lilley CH, Brauer AW, Bond JF et al. The major dog allergens, Can f 1 and Can f 2, are salivary lipocalin proteins: cloning and immunological characterization of the recombinant forms. *Immunology* 1997;**92**:577–586.
 39. Mattsson L, Lundgren T, Olsson P, Sundberg M, Lidholm J. Molecular and immunological characterization of Can f 4: a dog dander allergen cross-reactive with a 23 kDa odorant-binding protein in cow dander. *Clin Exp Allergy* 2010;**40**:1276–1287.
 40. Saarelainen S, Taivainen A, Rytkenon-Nissinen M, Auriola S, Immonen A, Mantjarvi R et al. Assessment of recombinant dog allergens Can f 1 and Can f 2 for the diagnosis of dog allergy. *Clin Exp Allergy* 2004;**34**:1576–1582.
 41. Rytkenon-Nissinen M, Saarelainen S, Randell J, Hayrinen J, Kalkkinen N, Virtanen T. IgE reactivity of the dog lipocalin allergen Can f 4 and the development of a sandwich ELISA for its quantification. *Allergy Asthma Immunol Res* 2015;**7**:384–392.
 42. Hilger C, Kuehn A, Hentges F. Animal lipocalin allergens. *Curr Allergy Asthma Rep* 2012;**12**:438–447.
 43. Saarelainen S, Rytkenon-Nissinen M, Rouvinen J, Taivainen A, Auriola S, Kauppinen A et al. Animal-derived lipocalin allergens exhibit immunoglobulin E cross-reactivity. *Clin Exp Allergy* 2008;**38**:374–381.
 44. Kamata Y, Miyanomae A, Nakayama E, Miyanomae T, Tajima T, Hoshi H. Characterization of dog allergens Can f 1 and Can f 2. 1. Preparation of their recombinant proteins and antibodies. *Int Arch Allergy Immunol* 2007;**142**:291–300.
 45. Madhurantakam C, Nilsson OB, Uchtenhagen H, Konradsen J, Saarne T, Hoggom E et al. Crystal structure of the dog lipocalin allergen Can f 2: implications for cross-reactivity to the cat allergen Fel d 4. *J Mol Biol* 2010;**401**:68–83.
 46. Nilsson OB, Binnmyr J, Zoltowska A, Saarne T, van Hage M, Gronlund H. Characterization of the dog lipocalin allergen Can f 6: the role in cross-reactivity with cat and horse. *Allergy* 2012;**67**:751–757.
 47. Juntunen R, Liukko A, Taivainen A, Narvanen A, Durand G, Kauppinen A et al. Suboptimal recognition of a T cell epitope of the major dog allergen Can f 1 by human T cells. *Mol Immunol* 2009;**46**:3320–3327.
 48. Liukko AL, Kinnunen TT, Rytkenon-Nissinen MA, Kailaanmaki AH, Randell JT, Maillere B et al. Human CD4+ T cell responses to the dog major allergen Can f 1 and its human homologue tear lipocalin resemble each other. *PLoS One* 2014;**9**:e98461.
 49. Ronka AL, Kinnunen TT, Goudet A, Rytkenon-Nissinen MA, Sairanen J, Kailaanmaki AH et al. Characterization of human memory CD4 T-cell responses to the dog allergen Can f 4. *J Allergy Clin Immunol* 2015;**136**:1047–1054.
 50. Kinnunen T, Taivainen A, Partanen J, Immonen A, Saarelainen S, Rytkenon-Nissinen M et al. The DR4-DQ8 haplotype and a specific T cell receptor Vbeta T cell subset are associated with absence of allergy to Can f 1. *Clin Exp Allergy* 2005;**35**:797–803.
 51. Roth-Walter F, Pacios LF, Gomez-Casado C, Hofstetter G, Roth GA, Singer J et al. The major cow milk allergen Bos d 5 manipulates T-helper cells depending on its load with siderophore-bound iron. *PLoS One* 2014;**9**:e104803.
 52. Roth-Walter F, Gomez-Casado C, Pacios LF, Mothes-Luksch N, Roth GA, Singer J et al. Bet v 1 from birch pollen is a lipocalin-like protein acting as allergen only when devoid of iron by promoting Th2 lymphocytes. *J Biol Chem* 2014;**289**:17416–17421.
 53. Stopkova R, Dudkova B, Hajkova P, Stopka P. Complementary roles of mouse lipocalins in chemical communication and immunity. *Biochem Soc Trans* 2014;**42**:893–898.
 54. Xu MJ, Feng D, Wu H, Wang H, Chan Y, Kolls J et al. Liver is the major source of elevated serum lipocalin-2 levels after bacterial infection or partial hepatectomy: a critical role for IL-6/STAT3. *Hepatology* 2015;**61**:692–702.
 55. Sola A, Weigert A, Jung M, Vinuesa E, Brecht K, Weis N et al. Sphingosine-1-phosphate signalling induces the production of Lcn-2 by macrophages to promote kidney regeneration. *J Pathol* 2011;**225**:597–608.
 56. Asimakopoulou A, Borkham-Kamphorst E, Tacke F, Weiskirchen R. Lipocalin-2 (NGAL/LCN2), a “HELP-ME” signal in organ inflammation. *Hepatology* 2015 Jun 5. doi: 10.1002/hep.27930.
 57. Alpizar-Alpizar W, Laerum OD, Illemann M, Ramirez JA, Arias A, Malespin-Bendana

- W et al. Neutrophil gelatinase-associated lipocalin (NGAL/Lcn2) is upregulated in gastric mucosa infected with *Helicobacter pylori*. *Virchows Arch* 2009;**455**:225–233.
58. Jha MK, Jeon S, Jin M, Lee WH, Suk K. Acute phase protein Lipocalin-2 is associated with formalin-induced nociception and pathological pain. *Immune Netw* 2013;**13**:289–294.
59. Otto GP, Hurtado-Oliveros J, Chung HY, Knoll K, Neumann T, Muller HJ et al. Plasma neutrophil gelatinase-associated lipocalin is primarily related to inflammation during sepsis: a translational approach. *PLoS One* 2015;**10**:e0124429.
60. Shields-Cutler RR, Crowley JR, Hung CS, Stapleton AE, Aldrich CC, Marschall J et al. Human urinary composition controls siderocalin's antibacterial activity. *J Biol Chem* 2015;**290**:15949–60.
61. Daure E, Belanger MC, Beauchamp G, Lapointe C. Elevation of neutrophil gelatinase-associated lipocalin (NGAL) in non-azotemic dogs with urinary tract infection. *Res Vet Sci* 2013;**95**:1181–1185.
62. Hsu WL, Lin YS, Hu YY, Wong ML, Lin FY, Lee YJ. Neutrophil gelatinase-associated lipocalin in dogs with naturally occurring renal diseases. *J Vet Intern Med* 2014;**28**:437–442.
63. Gouwelleuw L, Naude PJ, Rots M, DeJongste MJ, Eisel UL, Schoemaker RG. The role of neutrophil gelatinase associated lipocalin (NGAL) as biological constituent linking depression and cardiovascular disease. *Brain Behav Immun* 2015;**46**:23–32.
64. Candido S, Maestro R, Polesel J, Catania A, Maira F, Signorelli SS et al. Roles of neutrophil gelatinase-associated lipocalin (NGAL) in human cancer. *Oncotarget* 2014;**5**:1576–1594.
65. Ghosh M, Sodhi SS, Kim JH, Kim NE, Mongre RK, Sharma N et al. An integrated in silico approach for the structural and functional exploration of Lipocalin 2 and its functional insights with metalloproteinase 9 and lipoprotein receptor-related protein 2. *Appl Biochem Biotechnol* 2015;**176**:712–729.
66. Ruiz-Morales JM, Dorantes-Heredia R, Arrieta O, Chavez-Tapia NC, Motola-Kuba D. Neutrophil gelatinase-associated lipocalin (NGAL) and matrix metalloproteinase-9 (MMP-9) prognostic value in lung adenocarcinoma. *Tumour Biol* 2015;**36**:3601–3610.
67. Kudo-Saito C, Shirako H, Ohike M, Tsukamoto N, Kawakami Y. CCL2 is critical for immunosuppression to promote cancer metastasis. *Clin Exp Metastasis* 2013;**30**:393–405.
68. Jung M, Mertens C, Brune B. Macrophage iron homeostasis and polarization in the context of cancer. *Immunobiology* 2015;**220**:295–304.
69. Jung M, Weigert A, Tausendschon M, Mora J, Oren B, Sola A et al. Interleukin-10-induced neutrophil gelatinase-associated lipocalin production in macrophages with consequences for tumor growth. *Mol Cell Biol* 2012;**32**:3938–3948.
70. Jensen-Jarolim E, Achatz G, Turner MC, Karagiannis S, Legrand F, Capron M et al. AllergoOncology: the role of IgE-mediated allergy in cancer. *Allergy* 2008;**63**:1255–1266.
71. Warszawska JM, Gawish R, Sharif O, Sigel S, Doninger B, Lakovits K et al. Lipocalin 2 deactivates macrophages and worsens pneumococcal pneumonia outcomes. *J Clin Invest* 2013;**123**:3363–3372.
72. Makris K, Kafkas N. Neutrophil gelatinase-associated lipocalin in acute kidney injury. *Adv Clin Chem* 2012;**58**:141–191.
73. Bao G, Clifton M, Hoette TM, Mori K, Deng SX, Qiu A et al. Iron traffics in circulation bound to a siderocalin (Ngal)-catechol complex. *Nat Chem Biol* 2010;**6**:602–609.
74. Singh V, Yeoh BS, Xiao X, Kumar M, Bachman M, Borregaard N et al. Interplay between enterobactin, myeloperoxidase and lipocalin 2 regulates *E. coli* survival in the inflamed gut. *Nat Commun* 2015;**6**:7113.
75. Holden VI, Lenio S, Kuick R, Ramakrishnan SK, Shah YM, Bachman MA. Bacterial siderophores that evade or overwhelm lipocalin 2 induce hypoxia inducible factor 1 α and proinflammatory cytokine secretion in cultured respiratory epithelial cells. *Infect Immun* 2014;**82**:3826–3836.
76. Langelueddecke C, Roussa E, Fenton RA, Thevenod F. Expression and function of the lipocalin-2 (24p3/NGAL) receptor in rodent and human intestinal epithelia. *PLoS One* 2013;**8**:e71586.
77. Radauer C, Lackner P, Breiteneder H. The Bet v 1 fold: an ancient, versatile scaffold for binding of large, hydrophobic ligands. *BMC Evol Biol* 2008;**8**:286.
78. Seutter von Loetzen C, Hoffmann T, Hartl MJ, Schweimer K, Schwab W, Rosch P et al. Secret of the major birch pollen allergen Bet v 1: identification of the physiological ligand. *Biochem J* 2014;**457**:379–390.
79. Gomez-Casado C, Roth-Walter F, Jensen-Jarolim E, Diaz-Perales A, Pacios LF. Modeling iron-catecholates binding to NGAL protein. *J Mol Graph Model* 2013;**45**:111–121.
80. Chia WJ, Tan FC, Ong WY, Dawe GS. Expression and localisation of brain-type organic cation transporter (BOCT/24p3R/LCN2R) in the normal rat hippocampus and after kainate-induced excitotoxicity. *Neurochem Int* 2015;**87**:43–59.
81. La Manna G, Ghinatti G, Tazzari PL, Alviano F, Ricci F, Capelli I et al. Neutrophil gelatinase-associated lipocalin increases HLA-G(+) /FoxP3(+) T-regulatory cell population in an in vitro model of PBMC. *PLoS One* 2014;**9**:e89497.
82. Goetz DH, Holmes MA, Borregaard N, Bluhm ME, Raymond KN, Strong RK. The neutrophil lipocalin NGAL is a bacteriostatic agent that interferes with siderophore-mediated iron acquisition. *Mol Cell* 2002;**10**:1033–1043.
83. Breustedt DA, Chatwell L, Skerra A. A new crystal form of human tear lipocalin reveals high flexibility in the loop region and induced fit in the ligand cavity. *Acta Crystallogr D Biol Crystallogr* 2009;**65**:1118–1125.
84. Loch J, Polit A, Gorecki A, Bonarek P, Kurpiewska K, Dziedzicka-Wasylewska M et al. Two modes of fatty acid binding to bovine beta-lactoglobulin—crystallographic and spectroscopic studies. *J Mol Recognit* 2011;**24**:341–349.
85. Lascombe MB, Gregoire C, Poncet P, Tavares GA, Rosinski-Chupin I, Rabillon J et al. Crystal structure of the allergen Equ c 1. A dimeric lipocalin with restricted IgE-reactive epitopes. *J Biol Chem* 2000;**275**:21572–21577.
86. Niemi MH, Rytönen-Nissinen M, Janis J, Virtanen T, Rouvinen J. Structural aspects of dog allergies: the crystal structure of a dog dander allergen Can f 4. *Mol Immunol* 2014;**61**:7–15.
87. Bocskei Z, Groom CR, Flower DR, Wright CE, Phillips SE, Cavaggioni A et al. Pheromone binding to two rodent urinary proteins revealed by X-ray crystallography. *Nature* 1992;**360**:186–188.